

Contents lists available at [ScienceDirect](http://www.sciencedirect.com)

Journal of Food Engineering

journal homepage: www.elsevier.com/locate/jfoodeng

Encapsulation and controlled release of bioactive compounds in lactoferrin-glycomacropeptide nanohydrogels: Curcumin and caffeine as model compounds



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ARTICLE INFO

Article history:

Received 29 October 2015

Received in revised form

21 February 2016

Accepted 24 February 2016

Available online 27 February 2016

Keywords:

Caffeine
Curcumin
Whey proteins
Release behaviour

ABSTRACT

Curcumin and caffeine (used as lipophilic and hydrophilic model compounds, respectively) were successfully encapsulated in lactoferrin-glycomacropeptide (Lf-GMP) nanohydrogels by thermal gelation showing high encapsulation efficiencies (>90%). FTIR spectroscopy confirmed the encapsulation of bioactive compounds in Lf-GMP nanohydrogels and revealed that according to the encapsulated compound different interactions occur with the nanohydrogel matrix. The successful encapsulation of bioactive compounds in Lf-GMP nanohydrogels was also confirmed by fluorescence measurements and confocal laser scanning microscopy. TEM images showed that loaded nanohydrogels maintain their spherical shape with sizes of 112 and 126 nm for curcumin and caffeine encapsulated in Lf-GMP nanohydrogels, respectively; in both cases a polydispersity of 0.2 was obtained.

The release mechanisms of bioactive compounds through Lf-GMP nanohydrogels were evaluated at pH 2 and pH 7, by fitting the Linear Superimposition Model to the experimental data. The bioactive compounds release was found to be pH-dependent: at pH 2, relaxation is the governing phenomenon for curcumin and caffeine compounds and at pH 7 Fick's diffusion is the main mechanism of caffeine release while curcumin was not released through Lf-GMP nanohydrogels.

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1. Introduction

The demand for encapsulation systems continues to grow as the food industry needs to preserve the benefits of active compounds and deliver them at specific conditions. Encapsulation of bioactive compounds in food industry can be used to: i) preserve functional properties, ii) improve the stability of compounds with low solubility in relevant (mostly aqueous) media, iii) mask undesirable flavours, iv) enhance health benefits of food products (i.e. development of functional foods), v) control the release of bioactive compounds at desired time and specific target, and vi) increase the bioavailability of bioactive compounds (Davidov-Pardo et al., 2015; Gunasekaran et al., 2007; Huang et al., 2010; Kayitmazer et al., 2013; Livney, 2010). Despite the interesting and unique properties that encapsulation can bring to food industry, its use is still a challenge mainly due to the need of using GRAS (generally recognized as safe) materials for the development of

encapsulation systems.

Milk proteins are considered a vital macronutrient in food, offering the possibility of developing delivery systems for both hydrophilic and lipophilic bioactive compounds (Augustin and Oliver, 2014; Chen et al., 2006). Their biocompatibility, biodegradability, non-toxicity and ability to form hydrogels make them a relevant class of biopolymers to be used as vehicle of bioactive compounds (Fox, 2001), being one of the most promising systems used in food industry. Protein hydrogels are hydrophilic networks of swollen cross-linked polymers (Vermonden et al., 2012). The development of protein hydrogels at nano-scale is increasingly being studied for their attractive properties (e.g. ability to encapsulate different bioactive compounds, large surface area, response to environmental changes) in delivery systems (Oh et al., 2009; Yallapu et al., 2011). Lactoferrin (Lf) is an iron-binding glycoprotein with a isoelectric point around 8, found in various biological fluids of mammals. Lf is considered a multifunctional protein, playing several biological roles: antibacterial, antiviral, antifungal, anti-inflammatory, antioxidant and immunomodulatory (Bokkhim et al., 2013; Embleton et al., 2013; González-Chávez et al., 2009; Madureira et al., 2007).

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This protein is also marketed as a nutritional supplement with high potential for biopharmaceutical applications (Balcão et al., 2013). Glycomacropeptide (GMP) is an acid glycosylated peptide that occurs naturally in bovine milk within the whey fraction. This peptide is considered an abundant protein, comprising around 20% of the total protein in sweet cheese whey (Neelima et al., 2013; Gustavo Hermes et al., 2013; Thomä-Worringer et al., 2006; van Calcar and Ney, 2012). GMP is sold as a food ingredient and has an excellent safety record based on widespread supplementation of foods infant formulas, both using whey proteins (Brück et al., 2006). Furthermore, GMP has functional properties such as: emulsification and foaming ability and can act in the inhibition of cholera toxin binding, anti-cariogenic and preventing intestinal infections (Neelima et al., 2013; Gustavo Hermes et al., 2013). Protein nanohydrogels, produced by interaction between Lf and GMP, were developed and characterized in a previous work (Bourbon et al., 2015). Due to their small size (170 nm) and high stability at various values of temperature and pH, these systems promised to be an excellent vehicle for encapsulation of bioactive compounds.

Curcumin (diferuloyl methane), a yellow lipid-soluble polyphenol is present in the rhizome of turmeric (*Curcuma longa* L.) and is widely used as a colouring agent in food. A wide range of biological attributes of curcumin such as antioxidative, anti-inflammatory, antiangiogenic, antiamyloid anticancer, antimicrobial, wound-healing and hepatoprotective properties have been well reported (Bhawana et al., 2011). However curcumin's poor solubility, stability, and bioavailability in aqueous media limits its efficient use as a bioactive compound. Efforts are being done to increase the bioavailability of this bioactive compound in aqueous solution, e.g. through its encapsulation in various delivery systems such as nanoemulsions (Sari et al., 2015), nanocapsules (Kittitheeranun et al.) and nanoparticles (Li et al., 2013).

Caffeine (3,7-dihydro-1,3,7-trimethyl-1H-purine-2,6-dione), a white and water-soluble compound found in many plant species such as coffee and green tea, has received increasing attention in food and pharmaceutical industries due to its pharmacological properties, which comprise stimulation of the central nervous system, peripheral vasoconstriction, relaxation of the smooth muscle and myocardial stimulation (McLellan, 2006). Caffeine has frequently been used as a model compound, thus many formulations containing caffeine have been studied (Liédana et al., 2012; McLellan, 2006).

The present work aims at evaluating the ability of a protein-based nanohydrogel to encapsulate bioactive compounds with different water solubilities (curcumin as lipophilic compound and caffeine as hydrophilic compound) and evaluate their release mechanism through this matrix at different pH conditions, in an effort to reproduce at least partially the environment to be found during digestion, in view of future food applications.

2. Materials and methods

2.1. Materials

Lactoferrin-Lf was purchased from DMV International (USA) and Glycomacropeptide-GMP was kindly offered by Davisco Food International, INC. (Le Sueur, USA). To prepare the samples, it was used deionized water purified to a resistance of 15 MΩ, Millipore Corp. (France).

Hydrochloric acid was purchased from Panreac, Spain and

sodium hydroxide was obtained from Riedel-de Haen (Germany). Hydrophilic model compound, caffeine was purchased from VWR (USA) and Amicon® Ultra-0.5 centrifugal filter of 3 kDa and 8 kDa devices from, Millipore Corp. (Ireland) were used.

Lipophilic model compound, curcumin was purchased from Sigma–Aldrich, St. Louis and pure ethanol was purchased from Panreac (Barcelona, Spain). Fluorescein isothiocyanate (FITC) was purchased from Fluka (Germany). Standard marker proteins from PageRuler™ Broad Range Unstained Protein Ladder, Lot ##002252 was purchased from Thermo Scientific (Lithuania).

2.2. Encapsulation of bioactive compounds in nanohydrogels

Nanohydrogels were prepared as described by Bourbon et al. (2015). Briefly, 2.5 μM of Lf and 8.33 μM of GMP were dissolved separately, in deionized water at 25 °C. The pH values of biopolymer solutions were separately adjusted to 5.0, with 0.1 mol L⁻¹ of hydrochloric acid. Lf aqueous solution was added dropwise into GMP aqueous solution with gentle stirring until a final molar ratio (WR) of 1:7 (Lf:GMP) was reached.

2.2.1. Curcumin – lipophilic compound

Curcumin was used as lipophilic compound and a range of concentrations between of 0.005–0.18 mg mL⁻¹ previously dissolved in absolute ethanol was added to the Lf-GMP mixture. After gentle stirring for 30 min, the mixture of Lf-GMP with curcumin was subsequently heated at 80 °C for 20 min in a water bath (closed system) to obtain a homogeneously dispersed nanohydrogel.

The unbound curcumin was removed by centrifuging the sample at 12 000 g for 20 min, which pulls down only the undissolved curcumin. The pellet of curcumin was carefully dissolved in ethanol and curcumin was quantified, spectrophotometrically, at 425 nm (Li et al., 2013). The amount of curcumin loaded in nanohydrogels was calculated by deducting the amount recovered in the ethanol fraction from the total amount of curcumin used. These results were used to calculate the EE (Equation (1)).

2.2.2. Caffeine – hydrophilic model compound

As in the case of curcumin, an amount of caffeine (hydrophilic model compound) ranging from 0.02 to 3 mg mL⁻¹ previously dissolved in deionized water was gently added to the Lf-GMP mixture. After gentle stirring for 30 min, the mixture of Lf-GMP with caffeine was subsequently heated at 80 °C for 20 min in a water bath (closed system) to obtain a homogeneously dispersed nanohydrogel.

The unbound caffeine was determined after separating the nanohydrogels with encapsulated caffeine from the solution with free caffeine. The separation was performed using an Amicon® Ultra-0.5 centrifugal filter 3 kDa device (Millipore Corp., Ireland). Briefly, 0.5 mL of sample was added to the Amicon® and centrifuged at 14 000 g during 10 min. After centrifugation a filtrate with free caffeine and a concentrate with nanohydrogels with encapsulated caffeine were obtained. The free caffeine was evaluated spectrophotometrically at 272 nm, which corresponds to the maximum absorbance peak of caffeine (Bagheri et al., 2014b), and the amount of free caffeine was calculated using an appropriate calibration curve: $y = 6.37x + 0.09$ ($R^2 = 0.98$) being y the Absorbance and x the concentration of free caffeine (mg.mL⁻¹). The obtained values were used to calculate the encapsulation efficiency (EE) (Equation (1)).

$$\text{Encapsulation Efficiency \%} = \frac{\text{total amount of compound} - \text{free compound}}{\text{total amount of compound}} \quad (1)$$

where the *total amount of compound* is the initial concentration of bioactive compound added to the mixture Lf-GMP and the *free compound* is the concentration of compound that was not loaded to nanohydrogels. All the measurements were performed in triplicate.

2.3. Characterization of nanohydrogels after encapsulation with bioactive compounds

2.3.1. Fourier transform infrared (FTIR) spectroscopy

In order to confirm the incorporation of the bioactive compounds into Lf-GMP nanohydrogels, fourier transform infrared (FTIR) spectroscopy analyses were carried out with a Perkin Elmer 16 PC spectrometer (Perkin Elmer, Boston, MA, USA) equipped with an ATR probe in the wavenumber region of 600–4000 cm^{-1} using 16 scans for each sample. The samples were dried and then embedded in KBr pellets.

2.3.2. Fluorescence measurements

To evaluate protein hydrophobicity, 1-anilinoanthracene-8-sulfonic acid (ANS) a fluorescent probe was used. Stock solution of 3.6×10^{-3} M ANS was previously dissolved in 0.1 M phosphate buffer solution.

ANS solution was added to nanohydrogel solution until reach a saturation, which indicated that all hydrophobic parts of nanohydrogel were ligand to ANS probe. This saturation point was reached with a ANS final concentration of 0.08×10^{-3} M. ANS was added to nanohydrogels solutions after encapsulation with bioactive compounds and absorbance was measured.

Fluorescence measurements were performed at 25 °C using a spectrofluorimeter (Horiba Scientific) equipped with a standard thermostated cell holder. The excitation wavelength was 350 nm. Emission spectra were recorded between 300 and 360 nm with 1% attenuation, and fluorescence intensities were recorded every 0.5 nm. Excitation and emission slits were 15 nm. Data analysis of fluorescence peak was performed with Peak Fit 4.12 (SYSTAT Software Inc., Richmond, CA, USA) program.

2.3.3. Size distribution and polydispersity index

Nanohydrogels with encapsulated bioactive compounds were characterized in terms of size distribution (by number) and polydispersity index (PDI) using a Dynamic Light Scattering (DLS) apparatus (Zetasizer Nano ZS, Malvern Instruments, UK) equipped with a He–Ne laser at a wavelength of 633 nm. All measurements were performed at 25 °C. Each measurement of size and PDI was performed with a detection angle of 173° (Malvern, 2005). The results are given as the average \pm standard deviation of nine measurements.

2.3.4. ζ -potential

ζ -potential measurement was carried out at room temperature (25 °C) using a Zetasizer Nano ZS (Malvern Instruments, UK) in a folded capillary cell using a He–Ne laser-wavelength of 633 nm and a detector angle of 173° (Malvern, 2005). The measurements were made in triplicate, with three readings for each sample. The results are given as the average \pm standard deviation of nine

measurements.

2.3.5. Transmission electron microscopy (TEM)

TEM micrographs were conducted on a Zeiss EM 902A (Thornwood, N.Y., U.S.A.) microscope at accelerating voltages of 50 kV and 80 kV. The samples were prepared by dropping the solutions onto copper grids coated with carbon film and followed by natural drying (Bourbon et al., 2015).

2.3.6. Confocal laser scanning microscope (CLSM)

The distribution of curcumin and caffeine within the Lf-GMP nanohydrogels was examined by using a confocal laser scanning microscope (LSM 410, Carl Zeiss, USA). This technique allows visualization and characterization of structures not only on the surface, but also inside the particles, provided the material is sufficiently transparent and can be fluorescently labelled (Beirão da Costa et al., 2012).

Since curcumin is naturally fluorescent in the visible green spectrum, no further labelling of curcumin was needed (Gandapu et al., 2011). Nanohydrogels with encapsulated curcumin were mounted on a slide and visualized in the fluorescein isothiocyanate (FITC) wavelength at 488 nm.

To visualize caffeine distribution in protein nanohydrogels, caffeine was stained with FITC by the methodology described by Shu et al. (2010). Briefly, 0.0015 g of FITC solution (3 mg of colorant in 1 mL of 100 mM sodium carbonate solution, pH 9.3) was added to the caffeine solution and incubated at 20 °C for 1 h. Unattached colorant was removed by dialysis with a cut-off 100 Da. In order to identify the bioactive compounds, the laser was adjusted to green (FITC-labelled lactoferrin) mode which yielded an excitation wavelength of 488nm (green laser) and without fluorescence (bright-field). The superposition of the images obtained in these two channels allowed visualizing the protein nanohydrogels and the distribution of bioactive compounds, in the same image. All confocal fluorescence pictures were taken using a 40X objective.

2.4. Antimicrobial activity determination

The antibacterial activity of nanohydrogels, bioactive compounds (curcumin and caffeine) and nanohydrogels with bioactive compounds (curcumin and caffeine, respectively) was tested against two bacterial strains: *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative) by the disc agar diffusion test according to Wilkins et al. (1972). Briefly, the nanohydrogel (empty) and the nanohydrogel containing encapsulated bioactive compounds were absorbed in sterilized filter paper discs (of 0.6 cm in diameter) and placed on the lawn cultures of *S. aureus* and *E. coli*. The agar plates were incubated for 24 h at 37 °C and diameters of the inhibitory zone of clearance (cm) surrounding the discs were measured to estimate the antimicrobial activity. Sterile distilled water was used as control. In order to evaluate the antimicrobial activity of encapsulated caffeine and curcumin in Lf-GMP nanohydrogels, control solutions of free curcumin, free caffeine and Lf-GMP nanohydrogels without encapsulated compounds submitted at the same conditions (temperature and pH) used during the encapsulation procedure were tested.

2.5. Release kinetics of bioactive compounds from Lf-GMP nanohydrogels

The *in vitro* release kinetics of model compounds (caffeine and curcumin) was performed by a dialysis method (Azevedo et al., 2014; Rivera et al., 2015). Nanohydrogels with encapsulated bioactive model compounds (5 mL) were placed inside a dialysis membrane (molecular weight cut-off 8 kDa) that was subsequently placed into 40 mL of buffer solution (phosphate buffer for pH 7 and KCl–HCl buffer for pH 2) under magnetic stirring. At appropriate time intervals, 0.25 mL of supernatant were taken and 0.25 mL of fresh buffer were added to keep the volume of the release medium constant. The amount of caffeine and curcumin released from nanohydrogels was evaluated by measuring the absorbance at 272 and 425 nm, respectively (absorbance peak) (Elisa Biotech Synergy HT, Biotek, USA). All release tests were run at least in triplicate.

2.5.1. Release kinetics

The release profile of bioactive compounds from Lf-GMP nanohydrogel was evaluated using a kinetic model that accounts for both Fickian and Case II transport (linear superimposition model – LSM) effects in hydrophilic matrices (Azevedo et al., 2014; Berens and Hopfenberg, 1978; Rivera et al., 2015)

$$M_t = M_{t,F} + M_{t,R} \quad (2)$$

where M_t is the total mass released from the polymeric structure, $M_{t,F}$ and $M_{t,R}$ are the contributions of the Fickian and relaxation processes, respectively, at time t .

The Fickian process is described by:

$$M_{t,F} = M_{\infty,F} \left[1 - \frac{6}{\pi^2} \sum_{n=1}^{\infty} \frac{1}{n^2} \exp(-n^2 k_f t) \right] \quad (3)$$

where $M_{\infty,F}$ is the compound release at equilibrium and k_f is the Fickian diffusion rate constant. Equation (3) can be simplified using the first term of the Taylor series (Jeong et al., 1999).

Polymer relaxation (protein matrix) is initially driven by the swelling ability of the polymer and then related to the dissipation of stress induced by the entry of the penetrant and can be described as a distribution of relaxation times, assuming a first order-type kinetic equation (Berens and Hopfenberg, 1978).

$$M_{t,R} = \sum_i M_{\infty,R_i} [1 - \exp(-k_{R_i} t)] \quad (4)$$

where, M_{∞,R_i} are the contributions of the relaxation processes for compound release and k_{R_i} are the relaxation i th rate constants. For most cases, there is only one main polymer relaxation that influences transport and thus the above equation can be simplified using $i = 1$.

Therefore, the linear superimposition model for compound release from Lf-GMP nanohydrogels can be described by:

$$\frac{M_t}{M_{\infty}} = X \left[1 - \frac{6}{\pi^2} \exp(-k_f t) \right] + (1 - X) [1 - \exp(-k_R t)] \quad (5)$$

where X is the fraction of compound released by Fickian transport.

The experimental results were analysed by Equation (5) (linear superimposition model) in order to assess the transport mechanism involved for curcumin and caffeine release from nanohydrogels at pH 7.4 and 2.

2.5.2. Native polyacrylamide gel electrophoresis

In order to evaluate the integrity of nanohydrogel during the release experiments, a native-PAGE or “nondenaturing” gel electrophoresis, was performed. Native-PAGE analyses were carried out using the Mini-Protein II dual slab cell system equipped with a PAC 300 power supply (Bio-Rad Laboratories, Hercules, CA, USA). The resolving and stacking gel contained 10 and 4% of polyacrylamide, respectively. The gels were stained with 0.2% (w/v) silver nitrate (Chevallet et al., 2006). Standard marker proteins PageRuler™ Broad Range Unstained Protein Ladder was used to identify samples by their molecular weight.

2.6. Statistical analyses

The Equation (5) was fitted to data by non-linear regression, using a package of STATISTICA™ v 7.0 (Statsoft, Inc, USA). The Levenberg-Marquadt algorithm for the least squares function minimization was used. The quality of the regressions was evaluated on the basis of the determination coefficient, R^2 , the squared root mean square error, RMSE (i.e., the square root of the sum of the squared residues (SSE) divided by the regression degrees of freedom) and residuals visual inspection for randomness and normality. R^2 and SSE were obtained directly from the software. The precision of the estimated parameters was evaluated by the Standardised Halved Width (SHW %), which was defined as the ratio between the 95% Standard Error (obtained from the software) and the value of the estimate.

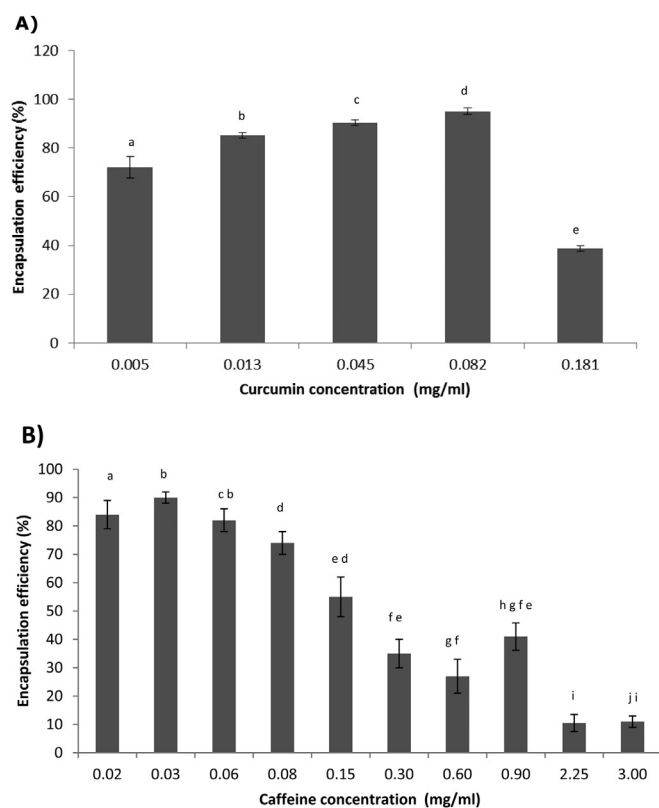


Fig. 1. Effect of curcumin concentration (A) and caffeine concentration (B) on encapsulation efficiency of Lf-GMP nanohydrogels, data are presented as mean \pm 95% confidence interval; values followed by different superscript letters are significantly different ($p < 0.05$).

3. Results and discussion

3.1. Bioactive compounds encapsulation

The physico-chemical properties of bioactive compounds (e.g. molecular weight, water solubility and chemical structure) are factors which will influence the selection of an efficient carrier to protect, transport and release them. In order to evaluate the effect of the bioactive compounds nature (i.e. hydrophilic and lipophilic) on the efficiency of encapsulation of Lf-GMP nanohydrogels, caffeine and curcumin were used as hydrophilic and lipophilic model compounds, respectively.

Different concentrations of curcumin and caffeine were evaluated and the concentration that allows the highest encapsulation efficiency of each bioactive compounds into nanohydrogels was determined (Fig. 1).

Results showed that the highest encapsulation efficiencies (EE) were obtained for concentrations of 0.082 mg mL^{-1} and 0.03 mg mL^{-1} for curcumin ($95.1 \pm 1.4\%$) and caffeine ($90.0 \pm 2.1\%$), respectively. The low solubility of curcumin in aqueous environments and its numerous health benefits make this lipophilic bioactive compound a target of numerous encapsulation studies. Different encapsulation methods and matrices have been tested (Ahmed et al., 2012; Bhawana et al., 2011; Das et al., 2010; Gandapu et al., 2011; Li et al., 2013; Sari et al., 2015). The ability of curcumin to establish interactions with proteins was also evaluated. The EE of this compound in protein matrices is around 90% depending on the system. As example, for zein nanoparticles prepared by electrohydrodynamic atomization it has been showed an EE around 90% (Gomez-Estaca et al., 2012), while Sneharani et al. (2010) using β -lactoglobulin particles to encapsulate curcumin obtained an EE around 96%. EE results obtained in the present work for Lf-GMP nanohydrogels are thus well within the range of values reported in the literature for other encapsulation systems.

EE values reported in literature for caffeine encapsulated in biopolymer matrices are around 83.6% for alginate-psyllium hydrogels (Belščak-Cvitanović et al., 2015), and 89.6% for particles composed by peptides obtained through hydrolysis of whey proteins and then cross-linked by transglutaminase (Bagheri et al., 2014a). The values obtained in the present work for caffeine in Lf-GMP nanohydrogels thus compare favourably with those reported in the literature (Ahmed et al., 2012; Bhawana et al., 2011; Das et al., 2010; Gandapu et al., 2011; Gomez-Estaca et al., 2012; Li et al., 2013; Sari et al., 2015).

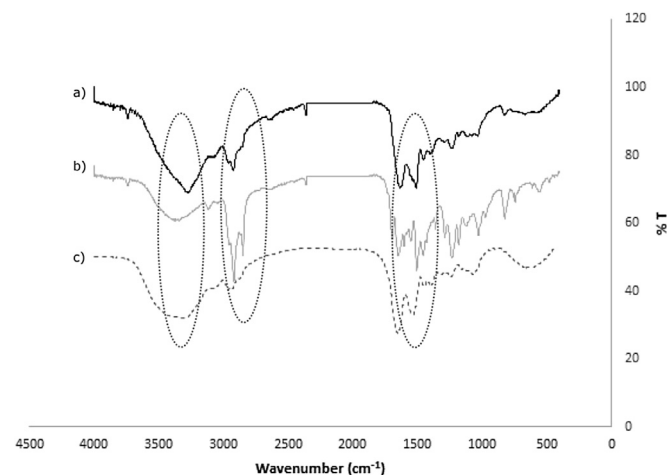


Fig. 2. FTIR spectra of a) curcumin encapsulated in Lf-GMP nanohydrogels, b) curcumin and c) Lf-GMP nanohydrogels without curcumin.

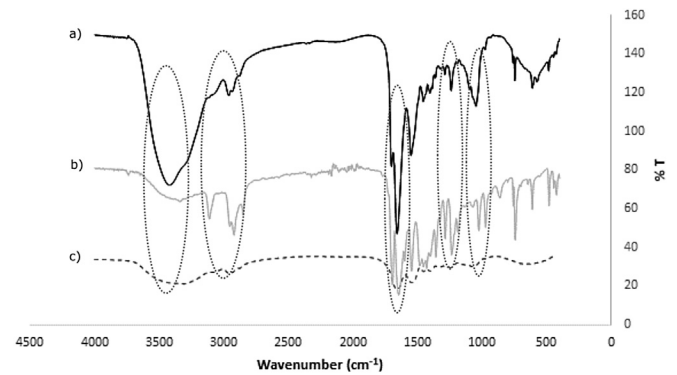


Fig. 3. FTIR spectra of a) caffeine encapsulated in Lf-GMP nanohydrogels, b) caffeine and c) Lf-GMP nanohydrogels without caffeine.

3.2. Characterization of nanohydrogels with encapsulated bioactive compounds

3.2.1. FTIR measurements

In order to evaluate the type of interaction between bioactive compounds and Lf-GMP nanohydrogels after the encapsulation, the FTIR spectra of bioactive compounds (curcumin and caffeine), Lf-GMP nanohydrogels, curcumin encapsulated in Lf-GMP nanohydrogels and caffeine encapsulated in Lf-GMP nanohydrogels were further evaluated and are shown in Figs. 2 and 3, respectively.

As expected, the FTIR spectrum of the mixture between curcumin and Lf-GMP nanohydrogels (Fig. 2b) contained peaks corresponding to both the components present. It is possible to observe a characteristic stretching band of curcumin (O–H) at 3508 cm^{-1} which differs from the peak observed for Lf-GMP nanohydrogels after the encapsulation, it was observed a shifted of peak to 3268 cm^{-1} , suggesting an interaction with protein matrix (Patra and Sleem, 2013; Yallapu et al., 2010). The peak at 1502 cm^{-1} , which corresponds to the ν (C=O), δ (CCC) and δ (CC=O) of curcumin undergoes a shift to 1512 cm^{-1} in the case of mixture with other components and this can be taken as an evidence of interaction between curcumin and Lf-GMP nanohydrogels. Also, the characteristic peak at 713 cm^{-1} of aromatic in plane bending of curcumin has shifted to 791 cm^{-1} after encapsulation. The region ranging between 3000 and 2800 cm^{-1} for curcumin encapsulated in Lf-GMP nanohydrogel spectrum corresponds to the CH_2 asymmetric and symmetric stretching vibrations. New bands are found in the range between 2500 and 3000 cm^{-1} . These results can suggest that the interaction of curcumin with Lf-GMP nanohydrogels is carried out through hydrophobic interactions of polyphenolic rings. Similar results have been reported in the reactions of resveratrol, genistein and curcumin with bovine serum albumin (Bourassa et al., 2010).

The presence of caffeine in Lf-GMP nanohydrogels is confirmed by the existence of characteristic bands of caffeine in Lf-GMP nanohydrogels (Fig. 3 a). The peaks at 1707 cm^{-1} , 974 cm^{-1} and 1359 cm^{-1} that correspond to C=O, C–C and C–H stretchings, respectively, indicate the presence of caffeine in Lf-GMP nanohydrogels (Kumar, 2010). It was also observed a presence of a new peak around 2990 cm^{-1} after the encapsulation of caffeine in Lf-GMP nanohydrogels, that can be due to C–H bonds of methyl ($-\text{CH}_3$) groups that are present in caffeine molecule. At 3500 cm^{-1} a higher peak is observed in spectra of caffeine encapsulated in Lf-GMP nanohydrogels, however this peaks are reported as bond vibrations of caffeine molecule (Paradkar and Irudayaraj, 2002). Moreover, it is possible to confirm the encapsulation of caffeine in Lf-GMP nanohydrogels due to notorious changes in amide I

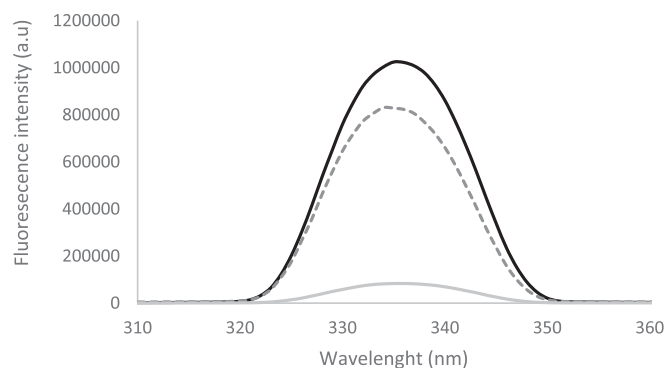


Fig. 4. Fluorescence emission of Lf-GMP nanohydrogels (—); caffeine encapsulated in Lf-GMP nanohydrogels (---) and curcumin encapsulated in Lf-GMP nanohydrogels (—), all samples were stained with ANS.

($1700\text{--}1600\text{ cm}^{-1}$), mainly C=O stretch, and amide II bands ($1600\text{--}1500\text{ cm}^{-1}$), C–N stretch coupled with N–H bending mode, suggesting a hydrophilic interaction between the caffeine and Lf-GMP nanohydrogel, since the main groups involved in these interactions are C=O, C–N and N–H (Bagheri et al., 2014a; Li et al., 2013).

3.2.2. Fluorescence measurements

Measuring the hydrophobicity of proteins is a useful technique to evaluate the protein binding sites. 8-anilino-1-naphthalenesulfonic acid (ANS) is a fluorescent probe that binds to hydrophobic sites of proteins (Alizadeh-Pasdar and Li-Chan, 2000). In order to evaluate the type of interaction between bioactive compounds and Lf-GMP nanohydrogels, surface hydrophobicity of nanohydrogels was measured after and before the interaction of nanohydrogel with bioactive compounds (Fig. 4).

ANS was applied to evaluate the hydrophobic surface of Lf-GMP nanohydrogels and monitor possible changes during the interactions with active compounds. Individual scans Lf-GMP nanohydrogels were performed to obtain the maximum peak of emission. It was observed that the wavelength of the maximum intrinsic fluorescence spectrum (λ_{max}) was at 336 nm, corresponding to ANS fluorescence (i.e. hydrophobic parts of nanohydrogels). As can be seen in Fig. 4, the interaction of caffeine and curcumin with Lf-GMP nanohydrogels decrease the peak area, indicating that the accessibility of hydrophobic residues in Lf-GMP nanohydrogels decrease. The peak area obtained for curcumin is much lower than caffeine, indicating that a higher number of hydrophobic interactions are established between curcumin and Lf-GMP nanohydrogels that in case of caffeine with Lf-GMP nanohydrogels.

3.2.3. Size and ζ -potential measurements

The values of size, Pdl and charge of Lf-GMP nanohydrogels after encapsulation of bioactive compounds are presented in Table 1.

It is possible to observe that the encapsulation of bioactive compounds resulted in a decrease ($p < 0.05$) of the size of nanohydrogels. This can be explained by the structural rearrangement of

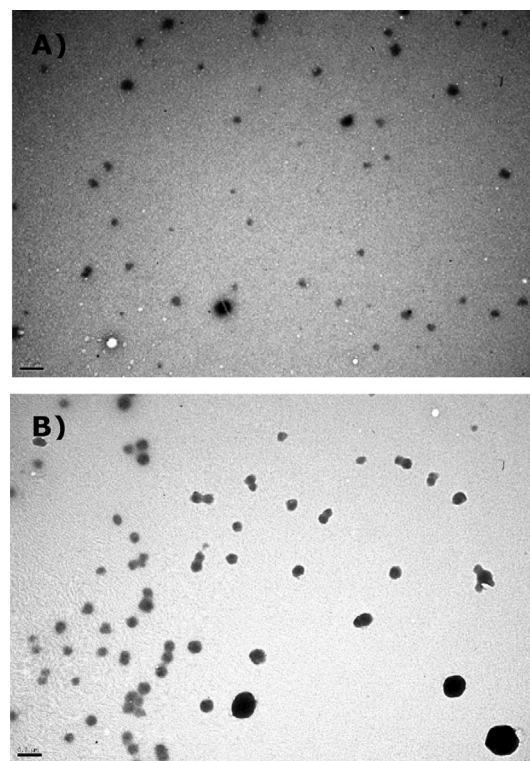


Fig. 5. Transmission electron microscopy images of Lf-GMP nanohydrogels with encapsulated A) caffeine and B) curcumin (scale bar – 0.2 μm).

Lf-GMP nanohydrogel after the encapsulation of the bioactive compounds, as reported by FTIR and fluorescence measurements. Moreover, it is important to mention that no statistically significant difference was observed between the size values obtained for Lf-GMP nanohydrogel with caffeine and Lf-GMP nanohydrogel with curcumin. This suggests that despite of the different nature and molecular weight of each bioactive compound, the size of the nanohydrogel particles was not affected. Pdl values are higher for Lf-GMP nanohydrogels with incorporated bioactive compounds, suggesting a decrease of homogeneity of the nanohydrogels. No significant differences were observed for the ζ -potential values after the incorporation of bioactive compounds, showing that the charge of the nanohydrogels is not affected, thus suggesting that electrostatic interactions are not involved in the encapsulation of the bioactive compounds and that their presence does not affect the superficial charge of nanohydrogel particles (i.e., possibly most of the bioactives are actually inside the gel matrix and not at its surface).

3.2.4. Morphology evaluation

The morphology of Lf-GMP nanohydrogels was evaluated by TEM (Fig. 5). TEM images show that Lf-GMP nanohydrogels with bioactive compounds encapsulated maintain the spherical shape with a solid dense structure and with a size around 120 nm and

Table 1

Effect of bioactive compounds encapsulation on nanohydrogel's properties (statistically significant differences in the values of the same column are identified by different superscript letters).

System	Size (nm)	Pdl	ζ -Potential (mV)
Lf-GMP nanohydrogels	170.02 ± 3.21^a	0.08 ± 0.01^a	-17.11 ± 1.45^a
Lf-GMP nanohydrogels + curcumin	112.03 ± 1.21^b	0.21 ± 0.04^b	-15.00 ± 0.75^a
Lf-GMP nanohydrogels + caffeine	126.03 ± 7.46^b	0.16 ± 0.02^b	-16.07 ± 1.84^a

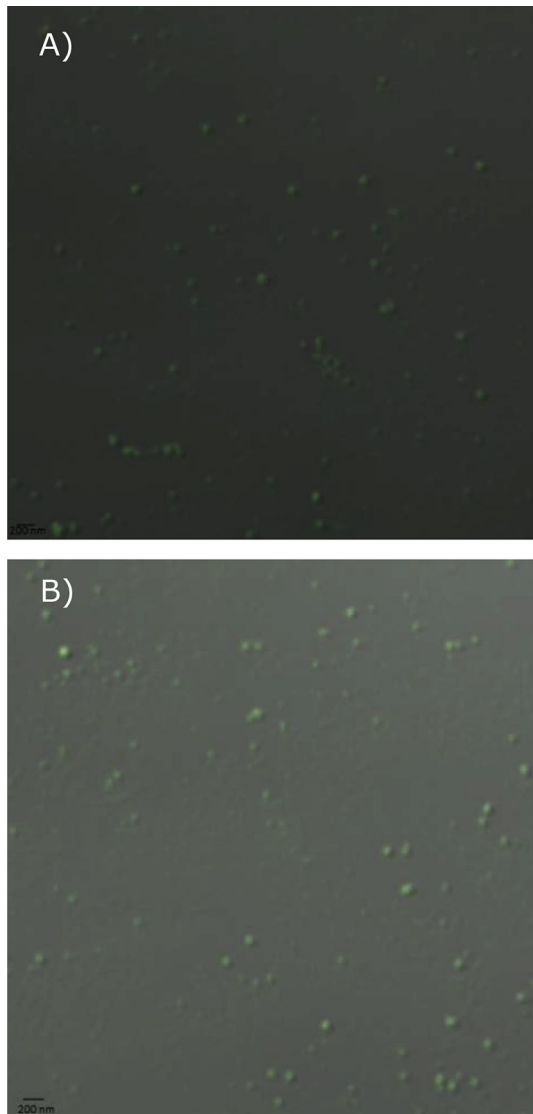


Fig. 6. Confocal laser scanning microscope images of Lf-GMP nanohydrogels with encapsulated A) curcumin and B) FITC-labelled caffeine; (scale bar – 200 nm).

125 nm for caffeine and curcumin, respectively. These results are in agreement with the size values obtained by DLS (Table 1).

In the present work, images obtained by CLSM allow identifying the distribution of each bioactive compound in Lf-GMP nanohydrogels. The green fluorescence channel was used to excite the bioactive compounds (Fig. 6).

Lf-GMP nanohydrogels without bioactive compounds were submitted to green laser light and no fluorescence signal was obtained (results not shown). When bioactive compounds were encapsulated in Lf-GMP nanohydrogels fluorescence was detected confirming the presence of those compounds inside the nanohydrogel structures. Round shaped and apparently compact structures could be observed, together with the green fluorescence of the bioactive compounds distributed into Lf-GMP nanohydrogel.

3.3. Antimicrobial activity determination

The disk diffusion assay was used to determine the antimicrobial activity of free and encapsulated compounds against *S. aureus* and *E. coli* (Fig. 7).

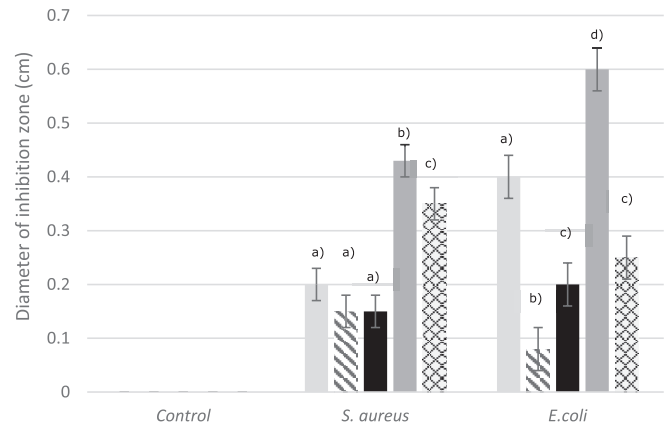


Fig. 7. Diameter of the inhibition zones (cm) for *S. aureus* and *E. coli* using: 0.08 mg mL⁻¹ of curcumin solution (□); 0.03 mg mL⁻¹ of caffeine solution (▨); Lf-GMP nanohydrogel solution (■); 0.08 mg mL⁻¹ curcumin encapsulated in Lf-GMP nanohydrogel solution (▩) and 0.03 mg mL⁻¹ caffeine encapsulated in Lf-GMP nanohydrogel solution (▩). Error bars represent the standard deviation obtained in triplicate experiments (statistically significant differences in the values of the same column are identified by different superscript letters).

Results showed that curcumin presents antimicrobial activity, as reported elsewhere (Bhawana et al., 2011; Zorofchian Moghadamtousi et al., 2014). Curcumin was found to be effective in inhibiting the growth of various Gram-positive and Gram-negative bacteria (Gutiérrez-Larraínzar et al., 2012). Results clearly showed that the antimicrobial activity significantly increases ($p < 0.05$) when curcumin is encapsulated in Lf-GMP nanohydrogels.

In fact, Lf-GMP nanohydrogels demonstrated to have antimicrobial activity against *S. aureus* and *E. coli*. One of the numerous properties of lactoferrin is its ability to exert a broad-spectrum primary defence activity against bacteria. This antibacterial activity is promoted by the destabilisation of the microorganism's cell membrane, which has different mechanisms for Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) organisms. For Gram-negatives, lactoferrin binds to porins present on the surface causing lipopolysaccharide release, and increasing bacterial fragility. In the case of Gram-positive bacteria, the membranes are disrupted by cationic residues and by hydrophobic residues in the N-terminus (Embleton et al., 2013).

The interaction of curcumin with Lf-GMP nanohydrogels demonstrated to result in a good synergy, fostering the antimicrobial properties of this new structure.

Caffeine showed a strong antimicrobial activity against *S. aureus*. This behaviour is due to the higher sensibility of Gram-positive bacteria to caffeic acid when compared to that of Gram-negative bacteria (Martínez-Tomé et al., 2011). This property makes of caffeine a natural food ingredient able to extend the shelf life of foods (e.g. cake, cookies, yoghurt) (Bagheri et al., 2014a; Liédana et al., 2012; Parwar et al., 2011). In line with results obtained for curcumin, also caffeine encapsulated in Lf-GMP nanohydrogels demonstrated to have a significantly ($p < 0.05$) higher antimicrobial activity when compared to caffeine alone.

The increase of antimicrobial activity of bioactive compounds after the encapsulation in Lf-GMP nanohydrogels can also be due to the fact that the small size of nanohydrogels increase their surface area-to-volume ratio in contact with microorganisms leading to enhanced particle surface reactivity (Romainor et al., 2014). Moreover, these results indicate that the synergy between bioactive compounds and the Lf-GMP nanohydrogels can result in a system with new functional properties.

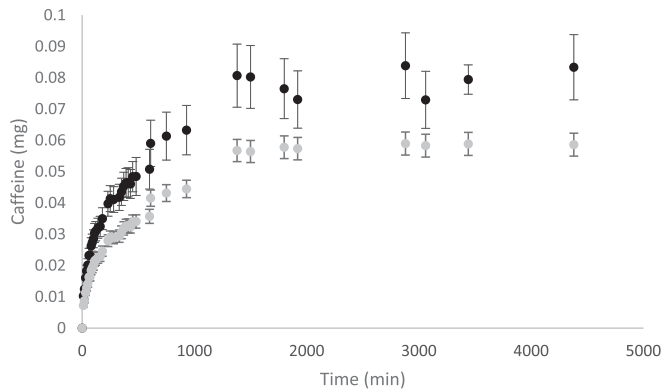


Fig. 8. Release kinetics of caffeine from Lf-GMP nanohydrogels at pH 2 (●) and pH 7 (●) at 37 °C.

3.4. Bioactive compounds release

A good understanding of the mechanisms involved in the release of bioactive compounds is extremely important when the design new carriers is desired, since it may allow predicting the carriers' behaviour during the production and/or consumption of a food product.

The experiments of bioactive compounds release from Lf-GMP nanohydrogels were conducted at 37 °C at two different pH values: 2 and 7. These conditions were used to simulate the release mechanisms of these bioactive compounds when subjected to digestion in the human gastrointestinal system.

Fig. 8 shows the release profile of caffeine from Lf-GMP nanohydrogels in contact with aqueous media at different values of pH.

Release profile of caffeine from Lf-GMP nanohydrogels revealed that at pH 2 a higher amount of caffeine was released from Lf-GMP nanohydrogels (Fig. 8). In a previous work it was observed that these nanohydrogels are sensitive to pH: the size and PDI of Lf-GMP nanohydrogels increased for pH values below 6 and above 8 and decreased when pH values ranged between 6 and 7 (Bourbon et al., 2015). Based in these observations it is likely that at pH 2 the nanohydrogels have a more relaxed, looser structure which possibly accounts for the easier and faster release of encapsulated compounds observed in Fig. 8 until an equilibrium state is reached. This behaviour can be due to various possibilities: i) bioactive molecules are on the surface of the carrier and are released faster than if they were entrapped, ii) conformational changes of protein nanohydrogels in contact with medium or iii) a stochastic

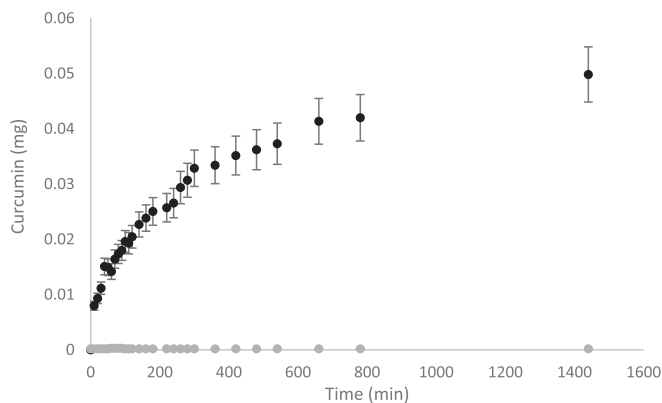


Fig. 9. Release kinetics of curcumin from Lf-GMP nanohydrogels at pH 2 (●) and pH 7 (●) at 37 °C.

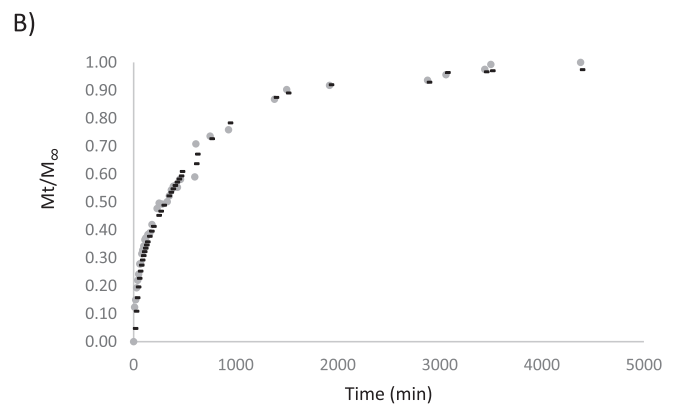
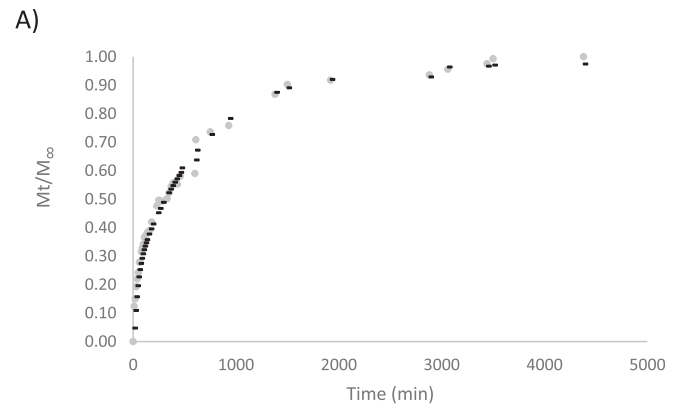


Fig. 10. Linear superimposition model (LSM) description of caffeine release at: (a) pH 2 and (b) pH 7 from Lf-GMP nanohydrogels, at 37 °C (experimental results (●); model-generated values (—)). M_t/M_∞ is the fraction of caffeine remaining in nanohydrogel until time (t).

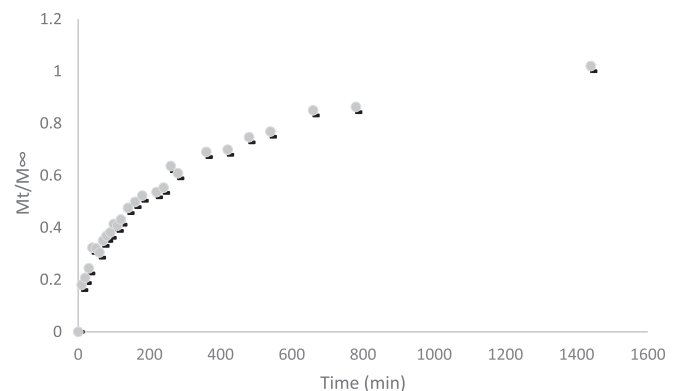


Fig. 11. Linear superimposition model (LSM) fitting of curcumin release data from Lf-GMP nanohydrogels at pH 2, 37 °C (experimental results (●); model-generated values (—)). M_t/M_∞ is the fraction of curcumin remaining in nanohydrogel until time (t).

phenomenon (related to Brownian motion), in which the penetrant flow is exclusively driven by a concentration gradient (Vesely, 2008).

Fig. 9 shows the release profile of curcumin from Lf-GMP nanohydrogels for the same conditions evaluated for caffeine (pH 2 and pH 7 at 37 °C).

Unlike caffeine, curcumin release from Lf-GMP nanohydrogels is clearly pH-dependent: while at pH 2 there is a clear release profile of curcumin from Lf-GMP nanohydrogels, at pH 7 curcumin was not released at all. The low solubility of curcumin in water at

Table 2
Results of fitting the Linear Superimposition Model (LSM) ($i = 1$) to experimental data of caffeine and curcumin release. Evaluation of the quality of the regression on the basis of RMSE and R^2 . Estimates' precision is evaluated using the Standardised Halved Width (SHW) % in parenthesis.

Bioactive compound	pH	RMSE	R^2	X	K_F (min^{-1})	K_R (min^{-1})
Caffeine	2	0.160	0.903	0.410 (48.20%)	0.001 (54.76%)	0.090 (26.56%)
	7	0.113	0.915	0.662 (45.23%)	0.087 (98.76%)	0.031 (87.33%)
Curcumin	2	0.105	0.965	0.341 (34.92%)	0.003 (67.34%)	0.182 (12.45%)

RMSE – Root mean square error.

X – The fraction of compound released by Fickian transport.

K_F – Fickian diffusion rate constant.

K_R – Relaxation rate constant.

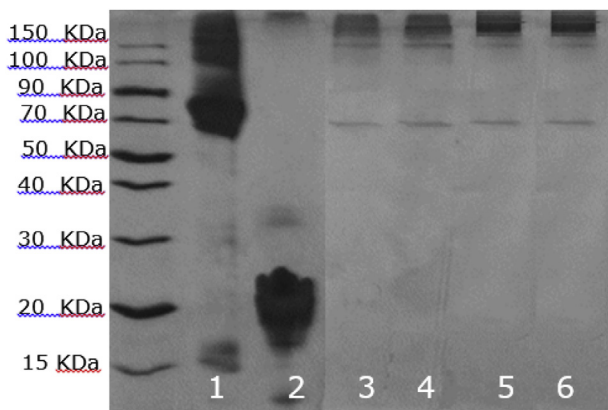


Fig. 12. Native-PAGE analysis of Lf (1), GMP (2), Lf-GMP nanohydrogels with caffeine encapsulated after the release experiments at pH 2 (3), Lf-GMP nanohydrogels with caffeine encapsulated after the release experiments at pH 7 (4), Lf-GMP nanohydrogels with curcumin encapsulated after the release experiments at pH 2 (5) and Lf-GMP nanohydrogels with caffeine encapsulated after the release experiments at pH 7 (6).

physiological pH ($0.0004 \text{ mg mL}^{-1}$ at pH 7.3) could be the reason of this behaviour. Patra and Sleem (2013) concluded that the release of curcumin was more triggered in acidic environment than under basic conditions. Also these authors observed that in acidic conditions curcumin is not stable and this may cause a weakening of interactions between curcumin and a protein matrix (in their case poly-L-lysine) thus facilitating curcumin release at acidic pH.

In this work, the description of experimental data obtained was made using the Linear Superimposition model (Equation (5)). This model has been chosen because it has been successfully used to describe the release mechanisms of different bioactive compounds from biopolymeric matrices (Azevedo et al., 2014; Pinheiro et al., 2013; Rivera et al., 2015). Fig. 10 and Fig. 11 shows the fitting of the linear superimposition model to experimental data of caffeine and curcumin released from Lf-GMP nanohydrogels, respectively.

Table 2 presents the regression analysis results of the LSM fitting for both compounds' release, showing that this model adequately describes the experimental data with a relatively good regression quality ($R^2 > 0.90$) and that almost all parameters were estimated with good precision.

This indicates that the release mechanism of the bioactive compounds through Lf-GMP nanohydrogels cannot only be described by Fick's diffusion but by both Fickian and Case II transport, with only one main relaxation of the Lf-GMP nanohydrogels.

X is defined as the Fick's diffusion contribution to the total release from the Lf-GMP nanohydrogels ($M_{\infty, F}/M_t$) and it can be observed from Table 2 that for both bioactive compounds, at pH 2 X is lower than 0.5, indicating that relaxation is the governing phenomena. One of the characteristics of protein matrices is the swellability (volume change) in response to external conditions such as pH or ionic strength (Chen, 2009). The behaviour of Lf-GMP

nanohydrogels was investigated at different pH conditions and it was observed that pH of the medium has a significant effect on the size of these nanohydrogels (Bourbon et al., 2015). Size of Lf-GMP nanohydrogels increase at pH 2 due to swelling behaviour of nanohydrogel at this condition. At pH 7, X is higher than 0.5 suggesting a higher contribution of Fick's diffusion for the release of the compound. In fact at this pH it was observed small sizes of Lf-GMP nanohydrogels, suggesting a decrease of matrix swelling (Bourbon et al., 2015). Relaxation rate constant (k_R) is higher for pH 2, supporting this hypothesis. As for the Fickian rate constant (k_F), as expected this parameter is higher for pH 7, reflecting the predominance of Fick's behaviour at that pH. At pH 2 the X value is lower for curcumin than it is for caffeine, suggesting that the relaxation mechanism is more predominant for the release of the lipophilic compound. This behaviour can be due to hydrophobic interactions established with curcumin (observed by FTIR and fluorescence measurements).

3.4.1. Electrophoresis

To ensure that Lf-GMP nanohydrogels are intact after the release measurements, a native electrophoresis was performed (Fig. 12).

Native electrophoresis clearly shows that Lf-GMP nanohydrogels are intact after the release experiments. This is shown by the presence of a high molecular weight band in all samples taken after release experiments (lanes 3 to 6). Moreover, it is also clear that no free proteins are present in these samples (Lf or GMP), suggesting that nanohydrogels are intact.

4. Conclusions

Lf-GMP nanohydrogels are able to incorporate bioactive compounds with different solubilities and with great potential to act as a controlled delivery system. Encapsulation of lipophilic and hydrophilic compounds showed high values of encapsulation efficiency, independent of the nature of the bioactive compound used. The encapsulation of bioactive compounds promoted an increase of antimicrobial activity when compared with active compounds in free solution.

The results of fitting the linear superimposition model to the experimental data of bioactive compounds release suggested an anomalous behaviour, with one main polymer relaxation and the bioactive compounds release was found to be pH-dependent.

Due to the bioactive, non-toxic nature and ability to encapsulate different bioactive compounds, Lf-GMP nanohydrogels are envisaged as a promising nanocarrier system to control the release of bioactive compounds for food and pharmaceutical applications.

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